Mucosal stimulation activates secretomotor neurons via long myenteric pathways in guinea pig ileum

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Reed DE, Vanner S. Mucosal stimulation activates secretomotor neurons via long myenteric pathways in guinea pig ileum. Am J Physiol Gastrointest Liver Physiol 292: G608–G614, 2007. First published September 28, 2006; doi:10.1152/ajpgi.00364.2006.—This study examined whether mucosal stimulation activates long secretomotor reflexes and so, how they are organized. The submucosa of in vitro full thickness guinea pig ileal preparations was exposed in the distal portion and intracellular recordings were obtained from electrophysiologically identified secretomotor neurons. Axons in the intact mucosa of the oral segment were stimulated by a large bipolar stimulating electrode. In control preparations, a single stimulus pulse evoked a fast excitatory postsynaptic potential (EPSP) in 86% of neurons located 0.7–1.0 cm anal to the stimulus site. A stimulus train evoked multiple fast EPSPs, but slow EPSPs were not observed. To examine whether mucosal stimulation specifically activated mucosal sensory nerve terminals, the mucosa/submucosa was severed from the underlying layers and repositioned. In these preparations, fast EPSPs could not be elicited in 89% of cells. Superfusion with phorbol dibutyrate enhanced excitability of sensory neurons and pressure-pulse application of serotonin to the mucosa increased the fast EPSPs evoked by mucosal stimulation, providing further evidence that sensory nerves were involved. To determine whether these reflexes projected through the myenteric plexus, this plexus was surgically lesioned between the stimulus site and the impaled neuron. No fast EPSPs were recorded in these preparations following mucosal stimulation whereas lesioning the submucosal plexus had no effect. These results demonstrate that mucosal stimulation triggers a long myenteric pathway that activates submucosal secretomotor neurons. This pathway projects in parallel with motor and vasodilator reflexes, and this common pathway may enable coordination of intestinal secretion, blood flow, and motility.

secretomotor reflexes; submucosa; secretion

INTESTINAL EPITHELIAL FLUID and electrolyte secretions contribute significantly to the secretions needed to maintain chyme in a fluid state (8). Rates of secretion will vary according to the physiological state of the intestine. For example, intestinal secretion can be increased eightfold following intake of a meal (8). Studies in the canine intestine demonstrated that tactile stimulation of the mucosa or distension of the intestine evoked fluid and electrolyte secretions that were inhibited by cholinergic antagonists (6). These responses were observed following extrinsic denervation of the bowel, suggesting that intrinsic neural reflexes were involved. The proximity of enteric neurons to the mucosa suggests that the enteric nervous system is poised to rapidly activate epithelial secretion in response to luminal stimuli.

Hubel (22) provided the first direct evidence of the involvement of enteric nerves in regulating mucosal ion secretion. In these experiments, electrical field stimulation of full-thickness rabbit intestine evoked a tetrodotoxin-sensitive ion secretion. Carey and colleagues (7) identified secretomotor neurons in the submucosal plexus as the primary source of this neural regulation. In this study, ion secretion was absent in preparations in which submucosal ganglia were removed. Morphological and immunohistochemical analysis in the guinea pig has revealed two populations of submucosal secretomotor neurons: 1) a cholinergic population that is immunoreactive for choline acetyltransferase (ChAT); and 2) a noncholinergic population that is immunoreactive for vasoactive intestinal peptide (VIP) (4, 38). These separate secretomotor populations work in concert to provide the cholinergic and noncholinergic regulation of secretion observed in functional studies (12).

Secretomotor neurons in the guinea pig submucosal plexus have been shown to participate in short reflex pathways (i.e., millimeters) (42) although submucosal neurons project for longer distances in the guinea pig colon (30). In these studies, mechanical stimulation of the mucosa activated a secretory response in mucosa/submucosa preparations (13, 36, 43). This stimulation released serotonin (5-HT) from mucosal enterochromaffin cells which activated submucosal intrinsic primary afferent neurons (IPANs) (13). Secretomotor neurons were subsequently activated, leading to ion secretion from the epithelium. In the guinea pig colon, distention-evoked secretory reflexes confined to the submucosal plexus have also been described (43). Thus the submucosal plexus contains the afferent and efferent elements that evoke local ion secretion in response to mucosal stimuli.

There has been considerable controversy concerning the existence of long secretomotor neural reflexes in the intestine. Both in vitro and in vivo studies have suggested that neural secretory reflexes in rabbit small intestine did not project greater than 4 mm (23). Other in vivo studies, however, have observed a temporal relationship between secretion and motility (19, 33), indirectly suggesting that secretion may be coordinated with long motility reflexes via neural pathways that project through the myenteric plexus. More recently, in vitro studies in guinea pig ileum have provided direct evidence that mucosal stimulation activates long enteric vasodilator reflexes (35) and that these pathways project through the myenteric plexus, raising the possibility that parallel secretomotor pathways may exist. Thus the aim of the present study was to examine whether mucosal stimulation activates a long descending neural pathway that innervates submucosal secretomotor neurons. We found evidence for such a reflex and describe the pathways involved.
METHODS

Adult Hartley guinea pigs (150–250 g) of either sex were anesthetized with isoflurane inhalation and killed by cervical transection and exsanguination. The abdomen was opened, and 30- to 40-mm segments of ileum were excised ~10 cm from the ileocecal junction and flushed of their contents. Segments were pinned in a Sylgard-lined (Dow Corning, Midland, MI) petri dish containing a physiological saline solution (in mM: 126 NaCl, 2.5 NaH₂PO₄, 1.2 MgCl₂, 2.5 CaCl₂, 5 KCl, 25 NaHCO₃, and 11 glucose), gassed with 95% O₂-5% CO₂, for tissue dissection. Drugs were added to the bath by superfusion unless otherwise stated. All procedures and studies were approved by the Animal Care Committee of Queen’s University.

In Vitro Preparation

A 20-mm segment of ileum was cut along the entire mesenteric border and pinned open flat with the mucosa facing upward. A 5- to 10-mm section of mucosa in the aboral portion of the segment was stripped away by gentle dissection to expose the underlying submucosa. The preparation was then pinned in a small organ bath (2–4 ml) with the exposed submucosa facing upward.

Electrophysiology

The preparation was continually superfused with physiological saline solution gassed with 95% O₂-5% CO₂ and maintained at a temperature of 35–36°C. To minimize movement of the muscularis externa so that intracellular impalements could be maintained, nicotine (3 μM) was added. In 15% of recordings [12/78 cells, predominantly in the 5-HT and phorbol dibutyrate (PDBu) studies] atropine (1 μM) was also added to the solution because some muscle movement was still evident despite the use of nicotine, as previously described (29, 35). Previous functional studies suggest that atropine does not significantly affect interneuronal pathways in the enteric nervous system of the guinea pig intestine (40). Intracellular recordings were made by use of glass microelectrodes filled with 2 M KCl. Electrode resistance ranged from 70 to 105 MΩ. Changes in membrane potential were monitored via an Axoclamp 2A amplifier and recorded with Axoscope 8 software (Axon Instruments).

The impaled neurons were characterized electrophysiologically by the shape of the action potential and by using a focal bipolar stimulating electrode place on adjacent submucosal ganglia or interganglionic fiber tracts to elicit synaptic inputs. A single pulse (0.8-ms duration) or a pulse train (10–20 Hz, 400–800 ms) was used and resulting fast excitatory postsynaptic potentials (EPSPs), slow EPSPs, and inhibitory postsynaptic potentials (IPSPs) were recorded. Cells were classified as S-type neurons when the action potential did not exhibit a prolonged afterhyperpolarization (>3 s) or a hump on the falling phase of the action potential, and they received large-amplitude fast EPSPs (21, 39). IPSPs also signified S neurons.

The mucosa was stimulated electrically with a large bipolar electrode with exposed stimulating feet of ~2–5 mm. The electrode was placed in contact with the tips of the villi, with the electrode feet lying perpendicular to the long axis of the preparation (Fig. 1). Responses could be elicited by a stimulus of 25–40 V (30 V was typically sufficient when the larger stimulating electrode was used). The mucosa was stimulated with a single pulse or a pulse train of 10–20 Hz for 400–800 ms.

In an additional series of experiments, pressure-pulse application of 5-HT (1 mM) was applied to the mucosa (Picospirter II, General Valve). The puffer pipette (5 μm diameter) was lowered until directly over part of the area being electrically stimulated. Two 100-ms pulses (10 psi) were applied to this area.

Surgical Denervation

To determine whether axons in the mucosa were being stimulated as opposed to current being spread through the tissue onto myenteric ganglia, the mucosa and submucosa in the oral portion of the preparation were lifted away from the underlying circular muscle. The mucosa/submucosa was then secured back over the circular muscle with small pins. Electrical stimulation was then applied to the mucosa and responses were recorded. In this preparation, the submucosa (with the attached mucosa) can be lifted quickly as a whole sheet from the underlying circular muscle by using jewelers forceps to grasp several points on one edge of the mucosa/submucosa, with the aid of a dissecting microscope ×70 magnification). Because these layers can be easily separated with this technique, removing the mucosa in this fashion does not add significantly to the dissecting time and thus does not alter the viability of the mucosa compared with control tissues. Our previous studies have also shown that removing the mucosa and submucosa in this fashion does not interfere with the integrity of the underlying myenteric plexus (29, 41).

The neural pathways that mediated responses evoked by mucosal stimulation were examined by studying the effects of surgical lesions between the stimulating and recording electrodes. The use of a high-powered dissecting microscope (×70 magnification) enabled the different layers of this tissue to be readily visualized, as previously described (35). In these flat-sheet preparations, the submucosa is relatively transparent and can be easily identified and distinguished from the underlying relatively opaque circular muscle. By lifting one edge of the submucosa free with jeweler’s forceps, the submucosa was severed by placing the lower blade of small dissecting scissors beneath the submucosa and cutting through it in a straight line across the preparation. In a similar fashion, the neural pathways in the myenteric plexus were severed by pinning the flat sheet preparation serosal side up and using jeweler’s forceps to free up one edge of a portion of the circular muscle. The blade of small dissecting scissors was positioned at this point, and the longitudinal muscle and a portion of the circular muscle were cut in a straight line across the preparation. This denervation technique is readily reproducible because the longitudinal muscle (with adherent myenteric plexus) is such a thin layer of tissue that it would not be inadvertently separated from the myenteric plexus and relatively thick circular muscle.

Drugs

Atropine, nicotine, 5-HT, PDBu, and hexamethonium (Hex) were purchased from Sigma-Aldrich.
Results

Intracellular recordings were obtained from 78 submucosal neurons. Mean resting membrane potential was 52.8 ± 1.1 mV. All neurons were classified as S-type based on previous criteria (see METHODS). Submucosal fiber tract stimulation (10–20 Hz, 400–800 ms) was examined in a subset of neurons and evoked IPSPs in 82% neurons (31/38). Previous studies have demonstrated that neurons that receive an IPSP are comprised almost entirely of noncholinergic secretomotor neurons (immunoreactive for VIP) (3, 16, 34). Although the presence of an IPSP was not examined in all neurons because of the risk of inducing muscle movement and dislodging the intracellular electrode, the high percentage of IPSPs in those neurons examined agrees with our previous work (34) and that of others (3, 18) that there is a selection bias of impaling submucosal neurons that have IPSPs (i.e., VIP immunoreactive) because they are located in the center of ganglia. Since VIP immunoreactive neurons function primarily as secretomotor neurons (5), the majority of neurons examined in this study were likely VIP secretomotor neurons.

Electrical Stimulation of the Mucosa

In control preparations, a single electrical stimulus pulse applied to the mucosa evoked a fast EPSP in 53 of 63 neurons (Fig. 2A). Fast EPSPs had an amplitude of 7.9 ± 0.7 mV and a latency of 27.3 ± 1.3 ms (range = 14–59 ms) (calculated for the first 44 of 53 neurons). In some neurons, action potentials were superimposed on the fast EPSPs. Subthreshold stimuli (i.e., insufficient to elicit fast EPSP with single pulse) applied as a stimulus train often evoked fast EPSPs (Fig. 2B). Hexamethonium (100 µM) reversibly blocked the fast EPSPs (Fig. 2C; n = 3). No slow EPSPs were recorded when a stimulus train was applied to the mucosa.

Examination of Neural Pathways

To establish the neural pathways that activate secretomotor neurons following mucosal stimulation, a surgical lesion was made through either the myenteric or the submucosal plexus (see METHODS). This lesion was made approximately equidistant from the stimulating and recording electrodes (Fig. 3A). No fast EPSPs were recorded following mucosal stimulation when the myenteric plexus was selectively lesioned (n = 4; Fig. 3, B and C). In contrast, lesioning the submucosal plexus did not abolish the fast EPSPs (mean amplitude = 4.5 mV; n = 3; Fig. 3, B and C).

Evidence for Activation of Myenteric AH Neurons and Mucosal Nerve Terminals

Mucosal denervation. To determine whether the fast EPSPs were due to stimulation of axon terminals in the mucosa, the mucosa/submucosa in the oral portion of the tissue was dissected free from the underlying muscle layer and then placed back in its original position (Fig. 4A). Recordings were obtained from denervated preparations (n = 3) and control preparations (n = 4). Only one preparation was obtained from each animal, and these were studied in an alternating fashion, i.e., normal, denervated, normal, denervated, denervated, normal, normal. In denervated preparations, a 30-V stimulus evoked a fast EPSP in only one of nine cells (Fig. 4B). Increasing the stimulus to 50 V evoked a fast EPSP in three of six cells, suggesting that with sufficiently high current pulses current can potentially spread by nonneural mechanisms and directly activate neurons in the myenteric plexus. Conversely, a 25–30 V stimulus evoked a fast EPSP in eight of nine cells from control preparations (Fig. 4C; P < 0.05).

Mucosal 5-HT stimulation. Further confirmation that electrical stimulation activated mucosal nerve terminals was sought by applying 5-HT via pressure-pulse application at the site of electrical stimulation as shown in Fig. 1. A stimulus train was applied to the mucosa to evoke fast EPSPs in the impaled neuron, and this stimulus strength was maintained for the...
duration of the experiment. To account for changes in amplitude and number of fast EPSPs evoked, the total area under all of the fast EPSPs was compared. Following the initial electrical stimulus, there was a 5-min control period after which the electrical stimulus was reapplied. There was no significant difference between the fast EPSPs evoked by the initial electrical stimulus compared with the fast EPSPs evoked by the stimulus applied after the control period (total area $= 228.2 \pm 56.9$ mV·ms; 5-min control period: total area $= 303.0 \pm 78.7$ mV·ms, respectively; $P < 0.05$) (Fig. 5).

**PKC stimulation.** Previous studies have shown prolonged stimulation of synaptic inputs at low frequency evokes a long-term (i.e., hours) increase in excitability of IPANs of the myenteric plexus but not interneurons or motoneurons (1, 10). Subsequent studies demonstrated that the effects of stimulation with the PKC agonist PDBu (1 $\mu$M) were indistinguishable from those obtained with low-frequency synaptic stimulation (25). Moreover, the actions of low-frequency synaptic stimulation on neuronal excitability were blocked by PKC inhibition (31). To determine whether myenteric IPANs were involved in the reflex observed in this study we exploited this selective action of stimulation with PDBu.

Intracellular recordings were obtained from submucosal neurons ($n = 3$) and baseline fast EPSPs evoked by mucosal stimulation with a pulse train (20 Hz, 400–800 ms) were obtained (Fig. 6). Response to electrical stimulation (20 Hz, 400–800 ms) of an adjacent ganglion in the submucosal plexus was also recorded. PDBu was then superfused for 4 min, and the electrical stimulation of the mucosa and submucosal ganglia were then repeated at 5 min following the superfusion by using the same stimulus parameters (Fig. 6). PDBu stimulation resulted in a marked increase in the area of evoked fast EPSPs in response to mucosal stimulation ($P = 0.011$). In contrast, fast EPSPs evoked by stimulation of submucosal ganglia were not affected, consistent with the evidence that PKC activation does not enhance synaptic transmission from interneurons.

**DISCUSSION**

Submucosal secretomotor neurons tightly regulate chloride secretion from the intestinal epithelium and are the final common neurons of secretomotor reflexes (11). Previous work in the guinea pig ileum has demonstrated that short secretomotor reflexes (i.e., 1–3 mm) exist within the submucosal plexus (13, 32, 36). The major finding of the present study was that mucosal stimulation activates long descending secretomotor reflexes that project through the myenteric plexus. These pathways would not only serve to prepare a distal segment of intestine for oncoming chyme but may also be a mechanism to coordinate fluid and electrolyte secretion with motility.

We found that 82% of the neurons we tested received an IPSP, suggesting that most of the neurons were VIP secreto-motor neurons (3, 16, 34). This selection bias is in keeping with our previous studies and those of others that demonstrate that intracellular recording from submucosal ganglia naturally selects for these neurons because they are preferentially located in the center of the ganglia where the easiest and most stable impalements are obtained (3, 34). Indeed, although we did not test the synaptic inputs to all neurons, this anatomical bias alone makes it highly probable that most of the neurons were noncholinergic VIP secretomotor neurons.

**Activation of Mucosal Nerve Terminals Triggers Secretomotor Reflexes**

Several experiments were conducted to ensure that the electrical stimulation of the mucosa activated mucosal afferents and that synaptic responses recorded in the submucosal plexus resulted from the selective activation of these nerve
terminals and were not due to current spread to the underlying myenteric ganglia. The most direct evidence that activation of mucosal axons was involved was provided by studies that examined the effects of mucosal stimulation in mucosa-dener- vated preparations. In these studies, synaptic responses were almost completely absent in denervated preparations whereas almost all control preparations responded. We cannot exclude the possibility that axons or cell bodies in the submucosal plexus were activated in normal preparations because the submucosa was also denervated with the mucosa, but previous studies support a role for mucosal axons. These studies of intracellular recordings from myenteric S neurons and IPANs have shown that when the mucosa is intact the excitability of these neurons is significantly enhanced (2, 27). They directly demonstrated that electrical stimulation initiates an action potential in a mucosal axon terminal that travels antidromically to

Fig. 4. Effect of mucosal lesioning on fast EPSP. A: schematic drawing of preparation used. Mucosa/ submucosa is lifted away from underlying circular muscle. It is then placed back into its original position and secured with pins. B: representative recording showing that electrical stimulation of mucosa in this preparation fails to evoke fast EPSP in submucosal neuron. C: summary of percentage of cells that had a fast EPSP in response to mucosal stimulation in lesioned preparations (n = 1/9) and in normal preparations (n = 8/9). Preparations were used in an alternating sequence. (Fisher’s exact test, P < 0.05)

Fig. 5. Effects of 5-HT applied to site of electrical stimulation. A: representative recording of protocol. Stimulus train was applied to evoke fast EPSPs in the impaled neuron. Following a 5-min control period, the same stimulus is reapplied, resulting in very little change in response. At 5 min following pressure-pulse application of 5-HT, the stimulus train evokes a larger response. B: summary of total area under all fast EPSPs evoked by the stimulus initially, after a 5-min control period, and 5-min following 5-HT application (n = 4). (ANOVA with Student-Newman-Keuls post hoc test, *P < 0.05)
the cell body before invading another axon process that activates other neurons in the reflex pathway and that the threshold for this activation is reduced when the mucosa is intact.

We also examined the effects of 5-HT on mucosal electrical stimulation because mucosal release of 5-HT can trigger peristaltic and secretory reflexes (13, 17, 20, 26, 36) by stimulating nerve terminals in the mucosa. In the present study, 5-HT application increased the amplitude and frequency of fast EPSPs evoked by electrical stimulation of the mucosa, implying 5-HT altered the mucosal nerve terminals which electrical stimulation was activating. However, 5-HT did not evoke fast EPSPs alone nor did it have a significant effect until 5 min after its application. The explanation for these findings is unclear but may be due to the relatively small volume of 5-HT applied to a limited surface area and the ensuing time lag for diffusion into the mucosa. The local effective concentration of 5-HT would also be modified by the serotonin-selective reuptake transporter.

We exploited the selective effect of PDBu on the excitability of AH neurons (25) to confirm that these neurons were involved in the electrophysiological responses elicited in our studies following mucosal stimulation (see Fig. 6). Although we could not sustain recordings for prolonged periods of time, we did observe that these responses persisted for at least 5 min after drug application. The IPANs of the myenteric ganglia belong to the AH electrophysiological class (9). Because these are the initiating neurons for enteric reflexes, our findings suggest that synaptic events and inflammatory mediators that induce prolonged excitability may not only alter motility but also lead to exaggerated secretory responses in concert.

**Neural Pathways in Secretomotor Reflex**

Lesioning the myenteric plexus blocked the reflex pathway whereas lesioning the submucosa had no effect, which established that this neural pathway activated by mucosal stimulation was mediated by the myenteric plexus. The essential role of the myenteric plexus in this reflex is supported by in vivo studies of mucosal secretion in the rat jejunum (24). In these studies cholera toxin applied in the lumen induced a net fluid secretion that was blocked when the myenteric plexus was ablated. The present study also demonstrates that these reflexes can project over long distances (i.e., at least 1 cm). It is likely that these longer projections involve polysynaptic pathways (29), and descending myenteric interneurons immunoreactive for ChAT/5-HT, ChAT/somatostatin, ChAT/VIP, or VIP/nitric oxide synthase have been described (14, 15, 28). In addition, long descending projections from myenteric IPANs or interneurons may also be involved (37). The possibility that ascending pathways in the myenteric plexus may also mediate mucosal activated secretomotor reflexes was not examined in this study, but such pathways may exist, given previous evidence for ascending myenteric pathways projecting to the submucosal plexus (35, 41).

In conclusion, this study demonstrates that long secretomotor reflexes project through the myenteric plexus in parallel with enteric vasodilator reflexes (35, 42). In vivo studies have reported a temporal relationship between spontaneous motility events and intestinal fluid and electrolyte secretions (19, 33). In anesthetized ferrets, cyclical ion fluxes associated with intestinal motility were preserved following vagotomy and sympa-
thet bloc, suggesting that this coordination involved intrinsic nerves. Our results suggest that motility and fluid and electrolyte secretory reflexes may be coordinated by pathways projecting in the myenteric plexus.

REFERENCES


